

RNA: Disulphide Bond Containing Oligodeoxyribonucleotide Duplexes Selectivity Studies

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Abstract

Selectivity studies of RNA: disulphide bond containing oligodeoxynucleotide duplexes using melting temperature (T_m) have been reported. The T_m data of the RNA/ DNA duplexes showed that duplex formed with disulphide bond containing oligodeoxynucleotide melted at higher temperature than that of RNA/ unmodified DNA duplex by 9.5°C. Even RNA /oligodeoxynucleotide with adjacent thiol groups formed more stable duplex compared to RNA/unmodified DNA duplex. Studies also showed that the selectivity of duplexes with disulphide bond was out performed duplexes with unmodified oligonucleotide.

Keywords

C-5 Thiopropyne Thymidine Substituted Oligonucleotides; T_m Studies; Selectivity; Disulphide Bond Containing Oligonucleotides

Introduction

Synthetic oligonucleotides have greater potential to become a new type of rationally designed therapeutic agent. These compounds could potentially interfere with the expression of selected genes through interaction with mRNA, genome DNA (Uhlmann et al., 1990 and Helene et al., 1990) or regulatory proteins. (Tuerk et al., 1990, Bock et.al., 1992, Wu et al., 1990, Harel –Bellan et al., 1989 and Bielinska et al., 1990) Antisense oligonucleotides recognize target mRNA sequence through Watson-Crick hydrogen bonding among A and T and G and C. This recognition is highly specific and should lead to development of less toxic and more site specific therapeutic agents (Stepenson et al., 1978). The use of oligonucleotides as antisense inhibitor of gene expression (Uhlmann et al., 1990 and Goodchild et al., 1990) and probes for RNA processes (Lamond, et al., 1989, Blencowe et al., 1989 and Barabino et al., 1989) requires high affinity for RNA.

The concept used in antisense technology is relatively straightforward. The antisense technology makes use of oligonucleotide sequence, complementary to a specific mRNA, which can inhibit its expression and then a blockade is induced in the transfer of genetic information from DNA to protein. The antisense oligonucleotides are commonly used both in the laboratory and clinic. In general, relatively short oligonucleotides (13-25 nucleotides) hybridize (at least in theory) to a unique sequence in the total pool of targets present in cells. However, nonspecific effects occur with phenotype which cannot be ignored. The use of phosphodiester oligonucleotides is limited as they are rapidly degraded by the intracellular endonucleases and exonucleases, usually via 3'-5' activity (Akhtar et al., 1991, Wickstrom et al., 1986 and Eder et al., 1991). In addition degradation products of phosphodiester oligonucleotides, dNMP oligonucleotides, may be cytotoxic and also exert antiproliferative effects (Vaerman et.al, 1997, Kara et.al., 1999, Koziolkiewicz et al., 2001 and Dias *et al.*, 2002). Deoxyribonucleotide phosphodiester oligonucleotides should therefore not be employed in antisense experiments. The use of oligonucleotides as antisense inhibitor of gene expression (Uhlmann et al., 1990 and Goodchild et al., 1990) and probes for RNA processes, (Barabino et al., 1989, Blencowe et al., 1989 and Lamond et al., 1989) requires high affinity and selectivity for RNA. So there are basically two main problems in making use of oligonucleotides as therapeutics—(i) degradation of oligonucleotides (ii) non-specific binding of oligonucleotides to the target mRNA, the first of which has been studied in details by making nucleases resistance phosphodiester bonds and also by other modifications (Dias et al., 2002), while the later doesn't. The C-5 propyne analogs of 2'-deoxycytidine has been shown to significantly enhance double helix formation with single strand RNA, relative to thymidine and 5-methyl-2-deoxycytidine (Froehler et al., 1992) to improve the

affinity of single strand ligand towards single strand RNA. Various other successful modifications have been carried out (Gryaznov et al., 1995, Jones, et al., 1993, Beaucage et al., 1993, Verma et al., 1993, Thuong et al., 1993, Sanghvi et al., 1994, Egholm et al., 1992, Nielsen, et al., 1990, Gryaznov et al., 1994, Aerschot et al., 1995, Frank Seela et al., 2004, Bentley et al., 2007, Owczarzy et al., 2011, Ittig et al., 2011 and Yan et al., 2012) Recently we have described, disulphide containing oligonucleotide stabilizes DNA: RNA structure (Kumar, 2012). Here in this paper, RNA/ disulphide bond containing oligonucleotide duplex formation and selectivity studies have been discussed.

Material and Methods

Synthesis of Oligonucleotides

The unmodified oligonucleotides (deoxy- as well as ribo-) and C-5 thiopropyne substituted thymidine with oligonucleotide were synthesized, deprotected, purified as described before (Chaudhuri et al., 1995) as well as base composition analysis of oligonucleotides. The preparation of disulphide bond containing oligonucleotide and purification has been made (Kumar, 2009). The duplexes having free thiol groups for studies were obtained by reducing the disulphide bond as described earlier (Kumar, 2009). A portion of duplex (R+2) was separately treated with 100 mM DTT overnight and dialyzed against water (4X2L) and finally dried thereafter and 500 μ L buffer was added. The resulting solution was kept at 4 °C overnight.

Thermal denaturation studies

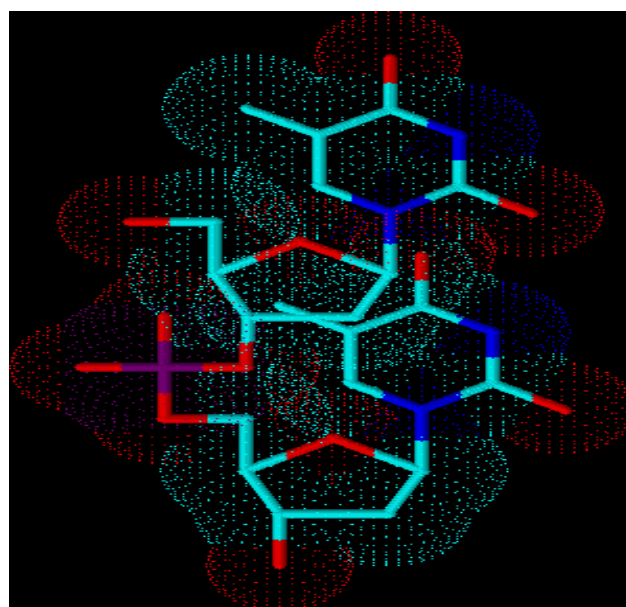
Solutions to the thermal denaturation studies of duplex contained one is to one ratio of a DNA oligomers and complementary RNA target oligomer (1.5 μ M each) in a buffer (100 mM, NaCl, 100 mM MgCl₂, 100 mM NaPIPES) at 7 pH were prepared. The mixtures were heated to 90 °C, then cooling down slowly to room temperature prior to the melting experiments, carried out in Teflon –Stoppered 1 cm path length quart cells under nitrogen atmosphere on a Varian Carry UV- VIS Spectrophotometer equipped with thermoprogrammer. Absorbance (260 nm) was monitored while temperature was raised from 10 °C at a rate of 0.5 °C/ min.

Results and Discussion

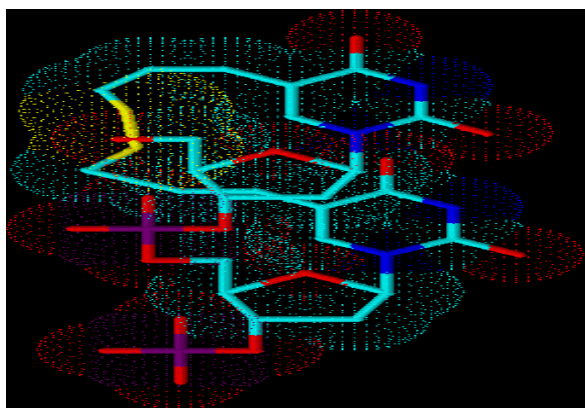
The oligonucleotides sequences synthesized are tabulated in Table-1. T_m data of the duplexes

determined is tabulated in Table –2. The ball and stick models of three types of dimers are shown in Fig.-1, a-c. In stick model of thymidine dimer, the methyl groups of thymidines are far apart whereas the –SH groups in the dimer of free thiols attached Thymidines, two thiol groups are quit close to each other. The polar surface areas of two thiols are overlap. The stick model of disulphide containing dimer shows that disulphide bond further brings two thiol groups near to each other and restricts the rotation of propyl bonds.

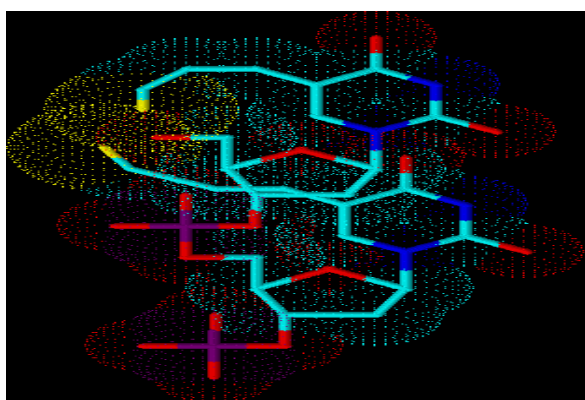
The duplexes are derived from oligonucleotides (1, 2 and 2a) and complementary single stranded RNA as well as mismatch with RNA sequences. T_m profiles of the duplexes are shown in Fig- 2, 3, 4. The T_m profiles of the duplexes are sharp and T_m is easily determined. The data (Table-1) has showed that melting temperatures (T_ms) of the duplexes [R(i-iv)+2] with disulphide bonded oligonucleotide were higher than that of the duplexes [R(i-iv)+1] with unmodified oligonucleotide. T_m of the duplex (R+2) was higher than that of (R+1) by 9.5 °C (Kumar, 2012). The oligonucleotide (2) showed selectivity for wobble base having RNA oligonucleotides in the range of 23.5-24.5 °C. Whereas the oligonucleotide (1) showed selectivity for wobble base with RNA oligonucleotides in the range of 16-19.0 °C. The oligonucleotide (2a) which contains reduced disulphide bond (i.e. two thiol groups) stabilized duplex better than unmodified but relatively less than to the disulphide with oligonucleotide. The selectivity of thiols containing oligonucleotide (2a) with wobble bases was comparable with unmodified oligonucleotides but relatively less than the disulphide including oligonucleotide (2).



a) STICKS STRUCTURE OF THYMIDINES DIMER



b) STICKS STRUCTURE OF DISULPHIDE WITH THYMIDINES



c) STICKS STRUCTURE OF FREE THIOLS ATTACHED TO ADJACENT THYMIDINES

FIG.1 BALL AND STICK MODELS OF DIMERS

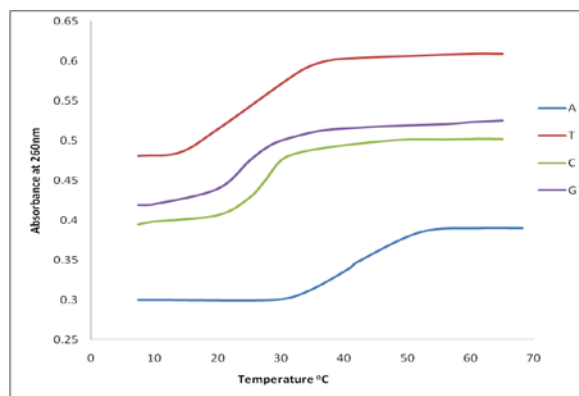
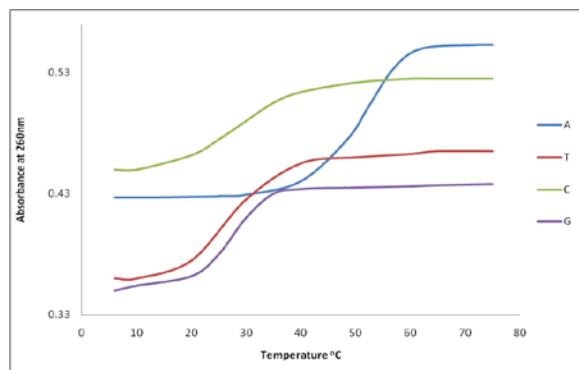
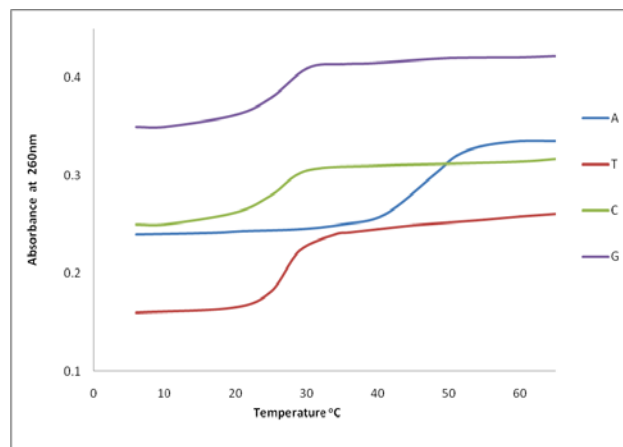
FIG. 2 T_m PROFILES OF UNMODIFIED OLIGODEOXYNUCLEOTIDE: OLIGORIBONUCLEOTIDES DUPLEXESFIG. 3 T_m PROFILES OF DISULPHIDE WITH OLIGODEOXYNUCLEOTIDE : OLIGORIBONUCLEOTIDES DUPLEXESFIG. 4 T_m PROFILES OF REDUCED DISULPHIDE WITH OLIGODEOXYNUCLEOTIDES: OLIGORIBONUCLEOTIDES DUPLEXES

TABLE 1 OLIGONUCLEOTIDES SYNTHESIZED

S.No	Oligonucleotide Sequence
RNA Seq.	3' AAGAAXGAAAAG 5'
	X= T,A,G,C
1	5' TTC TTCTTTTC 3'
2	S-S II 5' TTCT T TCTTTTC 3'
2a	HS SH II 5' TTCT T TCTTTTC 3'

TABLE 2 T_m DATA OF OLIGONUCLEOTIDE DUPLEXES

T _m ° C					
Duplexes	X=	A	T	C	G
R+1 (Figure-1)		42.5	26.5	24.0	23.5
Selectivity (S)			16.0	18.5	19.0
R+2 (Figure-2)		52.0	28.5	27.5	27.5
(S)			23.5	24.5	24.5
ΔT _m		09.5			
R+2a (Figure-3)		46.0	28.5	26.0	26.0
(S)			17.5	18.0	18.0
ΔT _m		03.5			

(S) Selectivity is defined as the difference between T_m of the correct oligonucleotide sequence and mismatch oligonucleotides sequences

Conclusion

Our T_m studies results have clearly showed that disulphide with oligonucleotide stabilizes the RNA/DNA duplex structure significantly more than unmodified oligonucleotide (Kumar, 2012). The disulphide with oligonucleotide also showed better selectivity for wobble base containing ribooligonucleotides than the unmodified oligonucleotide. The oligonucleotide with reduced disulphide bond (i.e. two free thiol groups) has stabilized RNA/DNA duplex relatively better than duplex with unmodified oligonucleotide. Hence both high stabilization and selectivity of RNA/ DNA duplexes are due to the presence of disulphide bond which stabilizes the conformation as shown in Fig-1. This stabilization and improved selectivity may result from the favorable geometry of disulphide bond present in the center of the oligonucleotide. The oligodeoxynucleotide, 2a which contains reduced disulphide bond (i.e. two thiol groups) stabilized duplex better than unmodified but less than disulphide bond containing oligonucleotide. Earlier Glick et al. (Glick et al., 1992 and Glick et al. 1991) have reported conformationally restricted DNA hairpin with disulphide cross link increasing the T_m by 21 °C relative to the wild type sequence. Froehler et al. (Froehler et al., 1992) have used oligodeoxynucleotides with C-5 propyne analogs of 2-deoxyuridine and 2-deoxycytidine for stabilization of duplexes. The stabilization of duplex in case of cross linked oligonucleotide is due to the restriction in flexibility and in case of oligodeoxynucleotides containing C-5 propyne analogs of 2-deoxyuridine and 2-deoxycytidine is due to π - π^* interactions. The thiopropyne modified oligonucleotide contains triple bonds to promote π - π^* interactions as well as a

disulphide bond between the two adjacent deoxyuridines to restrict the conformation.

References

- Aerschot, A.V., Verheggen, I., Hendrix, C., Herdewijn, P., "Oligonucleotides with 1, 5- Anhydrohexitol Nucleoside Building Blocks". *Angew. Chem Ind. Ed. Engel.*, 34, (1995): 1338-1339
- Akhtar, S., Kole, R., Juliano, R.L. "Stability of Antisense DNA Oligodeoxynucleotide Analogs in Cellular Extracts and Sera". *Life Sci.*, 49, (1991): 1793 –1801.
- Barabino, S. M. L., Spoart, B.S., Ryder, U., Blencowe, B.J., Lamond, A.I., "Mapping U2 snRNP:Pre-mRNA Interactions Using Biotinylated Oligonucleotides Made of 2'-OMe RNA". *EMBO Journal*, 8, (1989): 4171-4178.
- Beaucage, S. L., Iyer, R.P., "The Synthesis of Modified Oligonucleotides by the Phosphoramidite Approach and Their Applications". *Tetrahedron*, 49, (1993): 6123-6194.
- Bentley, J., Brazier, J.A., Fisher, J., Cosstick, R., "Duplex Stability of DNA.DNA and DNA.RNA Duplexes Containing 3'-S-Phosphorothiolate Linkages". *Org Biomol Chem.*, 21;5(22), (2007): 3698-702.
- Bielinska, A., Shivdasani, R.A., Zhang, L., Nabel, G.J., "Regulation of Gene Expression with Double-Stranded Phosphorothioate Oligonucleotides". *Science*, 250, (1990): 997-1001.
- Biochem. Biophys. Methods, 13, (1986): 97 –102.
- Blencowe, B. J., Spoart, B.S., Ryder, U., Barabino, S., Lamond, A.I., "Antisense Probing the Human U4/U6 snRNP with Biotinylated 2'-OMe RNA Oligonucleotides". *Cell*, 59, (1989): 531-539.
- Bock, L.C., Griffin, L.C., Lathans, J.A., Vermass, E.H., Toole, J.J., "Selection of Single-Stranded DNA Molecules that Bind and Inhibit Human Thrombin". *Nature*, 355 (6360), (1992): 564- 566.
- Chaudhuri, N.C., Kool, E.T., "Very High Affinity DNA Recognition by Bicyclic and Cross-Linked Oligonucleotides". *J. Am Chem Soc.*, 11, (1995): 10434-10442.
- Dias, N., Stein, C.A., "Antisense Oligonucleotides: Basic Concepts and Mechanisms, Molecular Cancer Therapeutics", Vol. 1, (2002): 347-355.
- "Displacement with a thymine-substituted polyamide", *Science*, 254, (1990): 1497-1500.
- Eder, P. S., DeVine, R.L., Dagle, J.M., Walder, J.A., "Substrate Specificity and Kinetics Of Degradation of Antisense Oligonucleotides by a 3' Exonuclease in Plasma". *Antisense Res. Dev.*, 1, (1991): 141 –151.
- Egholm, M., Buchardt, O., Nielsen, P.E., Berg, R.H., "Peptide Nucleic Acids (PNA) - Oligonucleotide Analogues with an Achiral Peptide Backbone", *J. Am. Chem. Soc.*, 14, (1992): 1895-1897.
- Frank Seela, F., He, Y., He, J., Becher, G., Kröschel, R., Zulauf, M., Leonard, P., "Base-Modified Oligonucleotides With Increased Duplex Stability: Pyrazolo[3,4-d] Pyrimidines Replacing Purines". *Methods in Molecular Biology*, 288 (2004): 165-186.

- Froehler, B. C., Wadwani, S., Terhorst, T.J., Gerrard, S.R., "Oligonucleotides Containing C-5 Propynyl Analogs of Deoxycytidine and 2'-Deoxycytidine". *Tetrahedron Lett*, 33(37), (1992): 5307-5310
- Glick, G.D., "Synthesis of a Conformationally Restricted DNA Hairpin". *J Org Chem.*, 56, (1991): 6746-6747.
- Glick, G. D., Osborne, S.E., Knitt, D.S., Marino Jr, J.P., "Trapping and Isolation of an Alternate DNA Conformation". *J Am Chem Soc.*, 114, (1992): 5447-5448.
- Goodchild, J., "Conjugates of Oligonucleotides and Modified Oligonucleotides: A Review of Their Synthesis and Properties". *Bioconjugate Chem.*, 1 (3), (1990): 165-187.
- Gryaznov, S., Chen, J., "P5' Phosphoramidates: Synthesis and Hybridization Properties". *J Am. Chem. Soc.*, 116, (1995): 3143-3144.
- Harel-Bellan, A., Brini, A., Ferris, D.F., Robin, P., Farrar, W.L., "In Situ Detection of a heat-Shock Regulatory /Element Binding Protein Using a Soluble Short Synthetic Enhancer Sequence". *Nucleic Acids Res.* 17 (11), (1989): 4077-4087.
- Helene, C., Toulme, J.J., "Specific Regulation of Gene Expression by Antisense, Sense and Antisense Nucleic Acids". *Biochem. Biophys. Acta*, 1049 (2), (1990): 99-105.
- Ittig D., Gerber, A.B., Leumann, C.J., "Position-Dependent Effects on Stability in Tricyclo-DNA Modified Oligonucleotide Duplexes". *Nucleic Acids Res.*, 39(1), (2011): 373-380.
- Jones, R. J., Swaminathan, S., Milligan, J.F., Wadwani, S., Froehler, B.C., Metteucci, M.D. "Oligonucleotides Containing a Covalent conformationally Restricted Phosphodiester Analog for High-Affinity Triple Helix Formation: The Riboacetal Internucleotide Linkage". *J. Am. Chem. Soc.*, 115(21), (1993): 9816-9817
- Kara, J., Duschinsky, R., "Inhibition of Thymidylate Kinase and DNA Synthesis in HeLa Cells by 5'-Deoxythymidine". *Biochim. Biophys. Acta*, 186, (1999): 223-225.
- Koziolkiewicz, M., Gendaszewska, E., Maszewska, M., Stein, C.A., Stec, W.J., "The Mononucleotide-Dependent, Nonantisense Mechanism of Action of Phosphodiester and Phosphorothioate Oligonucleotides Depends upon the Activity of an Ecto-5'-Nucleotidase". *Blood*, 98, (2001): 995-1002.
- Kumar, A., "C-5 Thiopropyne-Substituted Thymidine Containing Oligonucleotides: Modulation of Oligonucleotide Duplex, DNA/DNA and DNA/RNA Stability via Disulfide Bond". *The IUP Journal of Chemistry*, Vol. II, No. 4, (2009): 47-54.
- Kumar, A., "Disulphide Containing Oligonucleotide Stabilizes DNA: RNA Structure". *Chem Sci Trans.*, 1(1), (2012): 162-165.
- Lamond, A. I., Sproat, B.S., Ryder, U., Hamm, J., "Probing the Structure and Function of U2 snRNP with Antisense Oligonucleotides Made of 2'-OMe RNA", *Cell*, 58, (1989): 383-390.
- Nielsen, P. E., Egholm, M., Berg, R.H., Buchardt, O. "Sequence-Selective Recognition of DNA by Strand on the Stability and Discrimination of Mismatched Base Pairs of Duplexes". *J. Biosci.* 37(2), (2012): 233-241.
- Owczarzy, R., You, Y., Groth, C.L., Tataurov, A.V., "Stability and Mismatch Discrimination of Locked Nucleic Acid-DNA Duplexes". *Biochemistry*. 50(43), (2011): 9352-9367.
- Sanghvi, Y. S., Cook, P.D., "Carbohydrate Modification in Antisense Research", Sanghvi, Y. S., Cook, P.D.: *ACS Symp Series*, (1994): 580-1
- Stepenson, M. L., Zamecinik, P.C., "Inhibition of Rous Sarcoma Viral RNA Translation by a Specific Oligodeoxyribonucleotide". *Proc. Natl. Acad. Sci. (USA)*, 75 (1), (1978): 285-288.
- Thuong, N. T., Helene, C., "Triple Helix". *Angew Chem. Int. Ed. Eng.* 32, (1993): 666-690.
- Tuerk, C., Gold, L., "Systematic Evolution of Ligands by Exponential Enrichment: RNA Ligands to Bacteriophage T4 DNA Polymerase". *Science*, 249 (4968), (1990): 505-510.
- Uhlmann, E., Peyman, A., "Antisense Oligonucleotides: a New Therapeutic Principle". *Chem. Rev.*, 90 (1990): 544-584.
- Vaerman, J. L., Moureau, P., Deldime, F., Lewalle, P., Lammineur, C., Morschhauser, F., Martiat, P., "Antisense Oligodeoxyribonucleotides Suppress Hematologic Cell Growth through Stepwise Release of Deoxyribonucleotides". *Blood*, 90, (1997): 331-339.
- Verma, R. S. "Synthesis of Oligonucleotide Analogues with Modified Backbones", *Syn Lett.*, (1993): 621-637.
- Wickstrom, E., "Oligodeoxynucleotide Stability in Subcellular Extracts and Culture Media". *J.*
- Wu, H., Holecnberg, J.S., Tomich, J., Chen, J., Jones P.A., Huang, S.H., Calame, K.L., "Inhibition of in Vitro Transcription by Specific Double-Stranded Oligodeoxynucleotides". *Gene*, 89, (1990): 203-209.
- Yan, Y., Yan, J., Piao, X., Zhang, T., Guan, Y., "Effect of LNA- and OMeN-Modified Oligonucleotide Probes".